

EXHIBIT A

Targeting the Hedgehog Signaling Pathway with Small Molecules

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Abstract: In addition to the potential stem cells offer for regenerative medicine, they also rapidly are becoming a center of focus in oncology. There are several developmental pathways that are involved in the deregulated signaling in stem cells resulting in tumorigenesis. For example, aberrant activation of the *Hedgehog* (*Hh*) pathway has been associated with numerous malignancies including basal cell carcinoma, medulloblastoma, prostate, pancreatic and breast cancers. *In vivo* evidence suggests the antagonism of excessive *Hh* signaling may provide a route to unique mechanism-based anti-cancer therapies. This review summarizes recent developments in targeting cell-surface proteins and intracellular targets from the *Hh* pathway with small molecules. *Hh* signaling is triggered by lipid-modified *Hh* proteins that exert their activity *via* a series of transmembrane receptors (*Patched*, *Ptc* and *Smoothened*, *Smo*). *Smoothened* (*Smo*) is a 7-TM protein reported to be the most druggable target in the *Hh* signaling cascade. We further review several published programs geared towards identification and profiling of synthetic antagonists of *Smo*. Challenges and perspectives of this approach are also discussed.

Key Words: Hedgehog Pathway, Smoothened, Patched, Gli, Cell-Based Screening, Cyclopamine, Small Molecule Antagonists.

INTRODUCTION

Currently, there is an exponential growth in the interest towards stem cells in both academia and industry. In addition to the potential that stem cells offer for regenerative medicine, they are rapidly becoming the focus of tumorigenesis studies. It has been noted that there are several features shared between stem and tumor cells. These include self-renewal and formidable replication potential [1]. Also, it is believed that the early stage of oncogenesis involves acquisition of up to 4-7 mutations in a single cell accumulated during its lifespan. This hypothesis puts stem cells in the focus of attention as possible sources of various cancers. As expected, proliferation and differentiation of stem cells are closely regulated processes [1]. It has been suggested that the initial mutations in normal stem cells may lead to the expansion of aberrant stem cell population. This is followed by a series of transformations to yield transit amplifying cells followed by their conversion to the terminally differentiated cells of multiple types and formation of pre-malignant lesions. As a result, precisely compartmentalized differentiation gets disrupted and the dividing cells may leak into tissues that are "dormant" under normal conditions. Subsequent series of mutations and clonal selection lead to accelerated proliferation of these cells, decrease in their differentiation and subsequent metastasis *via* the invasion of mesenchyme [1]. A specific, mechanism-based modulation of this deregulated signaling at various check points is expected to offer treatments with better therapeutic windows than the conventional chemotherapy regimens that target proliferating cells nonspecifically.

Hedgehog (*Hh*) is one of the key signaling pathways determining the developmental outcome across a wide variety

of organisms, including mammals [2]. Over-stimulation of *Hh* signaling is involved in many different types of malignancies, including basal cell carcinoma (BCC), medulloblastoma [3], pancreatic cancer, small cell lung cancer, prostate cancer (PC), breast cancer and digestive tract tumors [4]. Early evidence for aberrant *Hh* signaling in tumorigenesis came from the identification of both the *PTCH* tumor suppressor gene in Gorlin's syndrome [5] and mutations in *SMO* leading to constitutive activation of the pathway [6]. Transcripts encoding the *Hh* ligands were present at high levels in all metastatic tumors and cell lines derived from metastases (reviewed in [1], Fig. (1)). Further, it has been demonstrated that *Hh* pathway activity and growth in these androgen-independent tumor cells are ligand-dependent. Both a neutralizing antibody directed against *Hh* ligands as well as a small molecule antagonist of the *Hh* pathway successfully modulated this aberrant activation resulting in complete regression and blockade of metastatic spread of subcutaneous PC3 and CWR22RV1 human PC xenografts.

Hh SIGNALING PATHWAY: RECOGNIZED SMALL MOLECULE TARGETS

Considering druggability and role in *Hh* signaling, there are several validated and suspected targets in the pathway for modulation with small molecules [7]. These targets represent different protein families and are both inter- and intracellular.

Hh signaling is triggered by the lipid-modified proteins that exert their activity *via* the series of transmembrane receptors (*Patched*, *Ptc* and *Smoothened*, *Smo*, Fig. (2A)). Three homologous *Hh* molecules have been found in vertebrates. These are *Desert* (*Dhh*), *Sonic* (*Shh*) and *Indian* (*Ihh*) *Hedgehog* proteins. The physiological effects of these in tissues were reported to be morphogenic, *i.e.* concentration-dependent, as evidenced by the formation of the spinal cord

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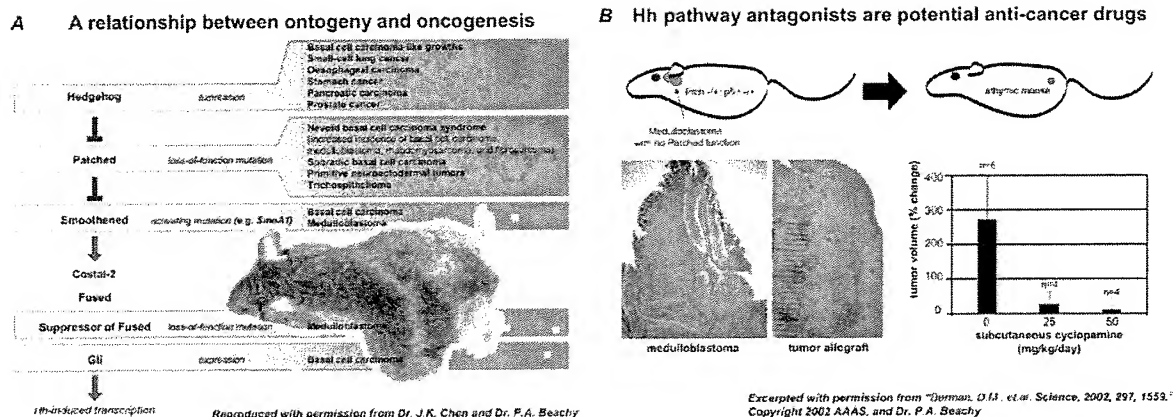


Fig. (1). (A): Role of various components of the Hh pathway in oncogenesis. (B): Hh pathway antagonist (cyclopamine) displayed efficacy in mouse medulloblastoma model.

and digits [8]. Two *Ptc* proteins, *Ptc1* and *Ptc2* have been identified to date. One of the specific features of *Hh* signaling involve kinase-mediated (*GSK3β*, *Cklα*, *PKA*) proteolysis and nuclear translocation of transcriptional factors (*Cubitus Interruptus*, *Ci* in *Drosophila* or *Gli1-3* in mammals) [9,10] resulting in cell proliferation [11,12]. A seven-pass transmembrane (7-TM) protein, *Smo*, is the key mediator of *Hh* signaling. *Smo* is a representative of the serpentine family of receptors [1]. It contains the extended *N*-terminal domain called CRD (cysteine-rich domain) and the 7-TM bundle. Despite of these similarities of *Smo* to GPCR's, neither endogenous *Smo* modulator nor the respective G-protein effector molecules have been identified to-date. Control of *Smo* activity in cells is realized via the 12-TM protein *Ptc*. *Ptc* in turn is negatively modulated by direct binding of *Hh* [13,14]. There are several proposed modes of *Ptc/Smo* interaction. A heteromeric receptor model has been suggested to rationalize biochemical evidence for their direct interaction [15]. However, based on the distinct *Ptc* and *Smo* localization *in vivo*, an alternative model has been introduced [16]. The same authors showed that both phosphorylation and sub-cellular location of *Smo* are important for pathway activation. There is considerable amount of evidence pointing to the catalytic character of *Smo* inhibition with *Ptc* [17].

The downstream effectors of *Hh* signaling have been studied to a lesser extent. Experiments in *Drosophila* led to the identification of a 155-residue protein *Ci* (*Ci155*). It forms the *Hh* signaling complex (HSC) with *Fu* (*Fused*, S/T kinase), *suppressor-of-fused* (*Su(fu)*) and a kinesin-like protein *Coastal-2* (*Cos-2*). In the absence of the *Hh* signal, the components are believed to be associated with microtubules. Kinase *Fu* was reported to be a critical component of the HSC, since mutations of *Fu* resulted in deregulation of all forms of *Ci* processing, ultimately leading to the loss of *Hh* signaling [1]. The carboxyl-terminal domain of *Fu* has been shown to associate directly with *Cos-2* [1]. Based on recent data, it is believed that a central role of *Fu* is the regulation of *Cos-2* and *Su(fu)* functions [18]. Although, this evidence suggests that *Fu* may be a viable target for antagonizing *Hh*

signaling, the mechanism that leads to *Fu* activation is not well understood. Part of the difficulty in identifying the substrates of *Fu* is the lack of an *in vitro* kinase assay [19]. Kinases *GSK-3β*, *Cklα* and *PKA* phosphorylate *Ci155* to yield its truncated 75-residue amino acid form (*Ci75*) that functions as a transcriptional repressor in the absence of a *Hh* signal [10]. It was suggested that *Hh* signaling results in activation of *Smo*, subsequent dissociation of the *Fu-Cos-2-Ci* complex from microtubules, blockade of *Ci155* processing and protein translocation to the nucleus, where it activates *Hh* target genes (Fig. (2)). In mammals, the regulatory functions of *Ci* are believed to be performed by the *Gli3* repressor protein, whereas two additional family members, *Gli1* and *Gli2* are the activators [20].

ALTERNATIVE TARGETS FOR Hh PATHWAY ANTAGONISM

Hydrophobic Modifications of the Hh Precursor Molecule

Recently, both the mechanism and specific components of *Hh* secretion have attracted considerable interest as potential points of intervention for a small molecule. It was reported that the *Hh* precursor molecule contains a *C*-domain with protease activity, whereas its *N*-terminal moiety serves as a signaling domain. During processing, the *N*-end signaling sequence is removed in an autocatalytic step followed by the modification of the *C*-terminus with cholesterol functionality [21]. In addition, an amino group of a conserved *Cys* at the *N*-terminus end is covalently modified with palmitoyl radical [22]. Data indicate that the enzyme *Rasp* is involved in this processing [23]. Both modifications are critical to the efficient signaling, although *Hh* proteins that lack either lipophilic moiety are still active at higher concentrations. Efficient membrane anchoring is believed to be responsible for the enhanced activity of the modified *Hh* [24]. In addition, this covalent processing could prevent *Hh* molecules from free tissue diffusion and uncontrolled distribution. Several additional elements of *Hh* transport and ex-

tracellular release rely on both cholesterol and palmitoyl post-modifications of the processed *Hh* molecule (*vide infra*).

Hh Transport

Blocking the release of *Hh* protein from a cell could be considered as an attractive path for altering *Hh* signaling. Several molecules have been characterized that perform this critical function. Protein *Dispatched* (*Disp*) is essential for *Hh* secretion as it specifically recognizes the cholesterol moiety of the processed *Hh* [25]. Similarly, enzyme *out-velu* (*itv*) involved in the synthesis of a specific heparine sulfate proteoglycans (HSPG's) is believed to promote transport of the cholesterol-modified *Hh* [26]. In addition, a mammalian LDL receptor-related protein (*Lrp*, a single-pass TM molecule) called *megalyn* was reported to interact directly with *Hh* [27]. Phenotypic evidence points out the similarities between *Hh* mutants and *megalyn* knock-outs in mice at the embryonic stage. The suggested function of *megalyn* is the regulation of cellular uptake of *Hh* proteins.

SMALL MOLECULES ANTAGONISTS OF THE Hh PATHWAY

Several research groups reported successful screening campaigns for sets of 10,000-140,000 molecules in a 96-well cell-based assay. Specifically, pathway activation was measured in Shh-LIGHT2 cells using a control medium. This cell line was derived from the NIH 3T3 cells by stable incorporation of a *Gli*-dependent firefly luciferase and constitutive *Renilla* luciferase reporters [28]. After incubation of the cells with small molecules, the luciferase activities were measured to identify actives (Fig. (2B)). Further profiling of the hits included whole-cell binding (293T cells transiently over-expressing *Smo*) and cell-free membrane-binding assays to determine molecules that bind to *Smo*. Chemical epistasis studies in cultured cell assays were used to determine the molecular level of action for the molecules signaling down-

stream of *Smo*. Specifically, the authors reported the use of i) a *Hh*-blocking antibody, ii) a *Smo* antagonist cyclopamine and its fluorescent derivatives, iii) a synthetic *Smo* antagonist CUR-61414 and iv) an adenylate cyclase/PKA activator forskolin that blocks *Hh* signaling, presumably by enhancing degradation of *Gli*'s [29]. The same authors also reported successful structure-activity relationship (SAR) studies for the series of compounds, confirming feasibility of hit optimization in the cell-based reporter assay.

Smo is the most "druggable" target in the *Hh* signaling cascade, as evidenced by identification of numerous chemical series of the pathway antagonists. This may be due to the endogenous regulation of *Smo* with a small molecule, presumably via a *Ptc*-mediated control of distribution for the ligand [30]. A *Veratrum* alkaloids jervine and cyclopamine were among the first identified *Hh* pathway antagonists, initially by following cases of lamb cyclopia (Fig. (2C)) [31]. It has been shown that cyclopamine inhibits *Smo* function by direct association with its heptahelical bundle causing pathway blockade and ultimately severe malformations in organ formation. Studies suggested the protein conformation as a primary determinant of *Smo* activity state. As evidenced by the binding assays, a small molecule agonist of the *Hh* pathway targets the same region in *Smo*, presumably inducing the active state of this TM receptor. Cyclopamine was reported to block activation of the *Hh* pathway and abnormal cell proliferation by affecting the equilibrium between active and inactive conformations of *Smo* [28]. A specific point mutation, tryptophane-to-alanine displacement in the last TM domain in *Smo* was found to be resistant to treatment of the pathway with cyclopamine and its derivatives. As a result, molecules that retain their activity against mutated *Smo* and/or downstream components of the pathway are of great interest for clinical development [29].

Using the assay described above, a number of chemically distinct *Smo* antagonists were identified and characterized mechanistically [32] (Fig. (3A,B)). These hits were reported

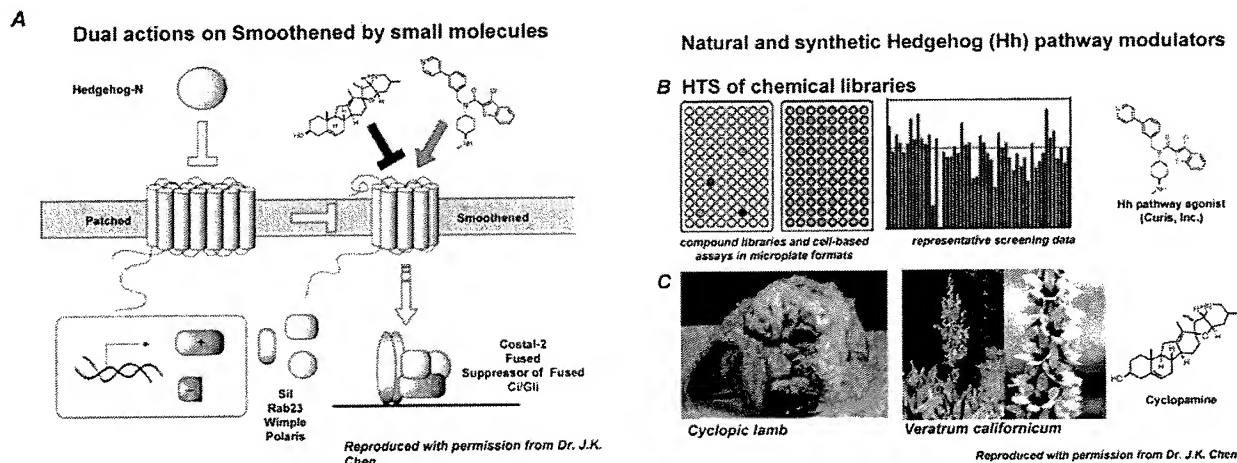
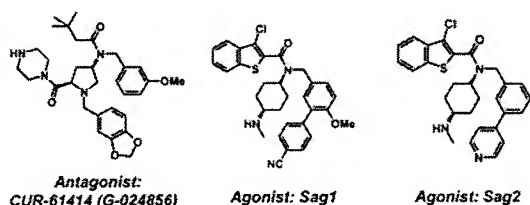
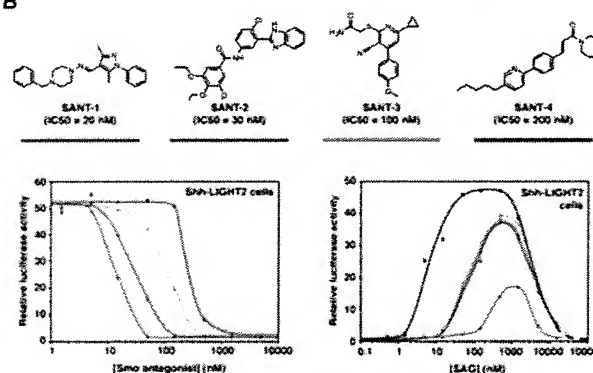


Fig. (2). (A): Key components of the Hh signaling cascade. (B): An illustration of a 96-well plate cell-based assay and structure of Hh pathway agonist designed by Curis.²⁸ Both cyclopamine and agonist derivatives were reported as useful tools in assay development and hit profiling; (C): Cyclopic lamb, an effect of a *Veratrum* alkaloid cyclopamine on a pregnant sheep.

A Small Molecule Modulators of Hh Pathway Developed by Curis, Inc.



B Smo Antagonists: different binding modes



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Fig. (3). (A): Small molecule antagonist and agonists developed by Curis, Inc. (B): Differential binding modes for synthetic antagonists (SANT's) of Hh pathway.

to overcome agonist-induced activation of the Hh pathway with different potencies. For example, a hydrazide derivative SANT-1 exhibited higher than expected (from its inhibitory activity in Shh-LIGHT2 cells) affinity towards *Smo*. In addition, it efficiently counter-acted agonist-induced stimulation of the pathway in cells.

Several antagonists failed to block association of the labeled cyclopamine and the *Smo*-expressing cells to the background level. It was suggested that these compounds alter *Smo* affinity towards cyclopamine and do not directly compete for binding. Selected, but not all identified compounds displayed comparable potencies in blocking pathway activation in both Shh-LIGHT2 and mutated *SmoA1*-LIGHT2 cells, as opposed to the cyclopamine derivatives suggesting conformational "plasticity" of *Smo* towards different chemotypes. Unusual behavior of the hydrazide derivative SANT-1 has been explained by its instability under the assay condition resulting in covalent binding to the target. This observation further accents the need for "drug-like" filtering of molecules comprising the Hh pathway screening set.

Notably, the small molecule *Smo* agonist displayed a bell-shaped concentration/Hh pathway activation curve peaking at 100 nM. Higher concentrations of the agonist furnished diminished potency not related to nonspecific cytotoxicity. This fact was explained by the combined effect of the agonist on both *Smo* and its downstream effector. The effect presumably reaches its peak at the agonist concentration that is optimal to insure for their interaction.

FURTHER STUDIES OF ANTAGONISTS OF Hh SIGNALING

A recent report described profiling of a new small molecule antagonist of the Hh pathway against a panel of sixty human cancer cell lines (NCI-60). Although compound structure has not been disclosed, it was stated that *ca.* 20% of the NCI-60 panel cells were sensitive to this molecule. Authors further proceeded to compare the gene complement of cells that displayed both resistance and sensitivity to the chemical. Further efforts are in progress to identify a panel

of genes whose expression might act as diagnostic indicators of Hh responsive tumors and to identify new molecules that show "fingerprint" profile similar to that for the confirmed Hh antagonist.

Cyclopamine displayed efficacy *in vivo* in the models of medulloblastoma. Specifically, it caused regression of murine tumor allografts and induced rapid death of cells from freshly resected human medulloblastomas, but not from other brain tumors, thus establishing a specific role for Hh pathway activity in medulloblastoma growth. Furthermore, analysis of 52 human breast carcinomas revealed staining of high intensity for *Gli1* when compared with adjacent normal tissue. Treatment with cyclopamine suppressed the expression of *Gli1* and the growth of the Hh pathway-activated breast carcinoma cells [33].

An antagonist molecule designed by Curis, Inc., CUR-61414 (Fig. (3A)) prevented proliferation and selectively induced death of the tumor cells, while not harming adjacent normal skin cells in two different models of BCC. The compound blocked elevated Hh signaling activity, suppressed proliferation and induced apoptosis of basaloid nests in the BCC model systems, whereas having no effect on normal skin cells [34].

CONCLUSION AND PERSPECTIVES

Despite of earlier concerns [35], Hh pathway modulators can be well tolerated in clinic, presumably due to the "silent" nature of the pathway in mature organisms. This is illustrated by the *in vivo* evidence that blocking the Hh signaling with cyclopamine across different species, while causing severe malformation in embryos, does not affect pregnant females [36]. With the availability of diverse potent small molecule antagonists, the long-term effects of Hh signaling blockade on the developed organism could be investigated in detail. In addition, relationships between the Hh and other developmental pathways (Wnt, Notch) could be unraveled. Small molecule modulators of the Hh pathway are expected to yield additional therapeutic benefits when compared to respective biological agents. For example, it is conceivable to

design chemicals that i) are resistant to the activating mutations in *Smo*, ii) affect intracellular targets on one or multiple levels of the pathway and iii) possess "tunable" efficacy *in vivo*. Further efforts in this area are required to identify both "optimal" druggable target(s) that yield robust *in vivo* response and the respective chemical agents that display the desired therapeutic window.

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